

A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA

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We describe the use of lentiviral vectors expressing small interfering RNAs (siRNAs) to knock down the expression of specific genes *in vitro* and *in vivo*. A lentiviral vector capable of generating siRNA specific for GFP after transduction of 293T-GFP cell lines showed no GFP fluorescence. Furthermore, no GFP-specific RNA could be detected. When eggs from GFP-positive transgenic mice were transduced with lentivirus-expressing siGFP virus, reduced fluorescence could be seen in blastocysts. More interestingly, pups from F₁ progeny, which expressed siGFP, showed considerably diminished fluorescence and decreased GFP. We propose that an approach of combining transgenesis by lentiviral vectors expressing siRNAs can be used successfully to generate a large number of mice in which the expression of a specific gene(s) can be down-regulated substantially. We believe that this approach of generating “knockdown” mice will aid in functional genomics.

gene silencing | transgenesis | functional genomics

Availability of the complete genome sequence of mouse and human, combined with extensive microarray data, has generated an abundance of biological information. However, methods to analyze the function of novel genes still remain laborious, time-consuming, and expensive. One of the most successful and informative methodologies involves the generation of gene knockout mice by homologous recombination. Because the use of antisense RNA to study gene function *in vitro* has often been unpredictable, the advent of RNA interference (RNAi) has introduced a new tool for deciphering gene function by inducing posttranscriptional gene silencing (1).

Use of RNAi by means of long double-stranded RNAs (>50 bp) is limited in mammalian cells because of the induction of a nonspecific inhibitory response resulting from the IFN pathway (2). This limitation was overcome by the finding that small interfering RNAs (siRNAs) that mimic intermediates in the RNAi pathway can specifically silence genes in mammalian cells without activating the nonspecific IFN pathway (3).

Recently several approaches have been described for generating loss-of-function phenotypes in mammalian systems by using RNAi (4–6). However, all these approaches have limited applications and are especially not applicable for generating a long-term silencing effect *in vivo*. To overcome these limitations, we have designed a lentiviral vector system capable of expressing siRNA in various mammalian systems. We have found that the lentivirus system can express integrated siRNA efficiently in a wide variety of cell lines and primary cells both *in vitro* and *in vivo*. More importantly, we demonstrated that a lentivirus vector system capable of expressing siRNA can efficiently transduce preimplantation mouse embryos. Furthermore, the resulting progeny expressed siRNA and showed reduced expression of specific genes. Here we report the development of the methodology for gene silencing using lentiviral vectors expressing siRNA.

Materials and Methods

Design and Cloning of siRNA Cassettes. The human H1-RNA promoter was cloned into pBluescript SK (Stratagene) by using

the primers CTGACGTCATCAACCCGCTCCA (forward) and CATACGTATTCGAATCTAGACACCAGAGTATGTCTTGAA (reverse). The design of the reverse primer incorporates *Bgl*II and *Hind*III sites juxtaposed on the transcriptional start site into which DNA sequences containing siRNA hairpins can be cloned. siRNA oligonucleotides were designed that contained a sense strand of 19 (p53) or 20 (GFP) nucleotide sequences followed by a short spacer (TTCAAGAGA), the reverse complement of the sense strand, and five thymidines as an RNA polymerase III transcriptional stop signal. Forward and reverse oligos for GFP and p53 were GFP forward (GATCCCCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCAGG-GTCAGCTTGCTTTTTTGAAA), GFP reverse (AGCTTTTCCAAAAAGCAAGCTGACCCTGAAGTTCTCTCTTGA-AGAACTTCAGGGTCAGCTTGCGGG), p53 forward (GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTA-GATTACCACTGGAGTCTTTTTTGAAA), and p53 reverse (AGCTTTTCCAAAAAGCAAGCTCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCGGG). Oligos were annealed and cloned into the *Bgl*II–*Hind*III site. For cloning into lentivectors, the complete human H1-RNA promoter plus the siRNA cassette was PCR-amplified by introducing *Xba*I sites both upstream and downstream of the sequence and cloned into a unique *Nhe*I site of the 3′ LTR of a lentiviral vector containing cytomegalovirus (CMV)-GFP (7, 8). This CMV-GFP cassette was deleted to generate LV-siGFP.

Transfection. 293T cells were cotransfected with a plasmid expressing GFP together with a plasmid expressing siRNA specific for GFP (siGFP) or human p53 (siHp53) in a ratio of 1/5 by using Fugene (Roche, Indianapolis).

Lentiviral Vector Production. Recombinant lentiviruses were produced by transient transfection in 293T cells using the calcium-phosphate method as described (7, 9, 10). Infectious lentiviruses were harvested at 48 and 72 h posttransfection and filtered through 0.22- μ m-pore cellulose acetate filters as described (7, 9, 10). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 \times g) and subsequently purified on a sucrose 20% gradient (2 h at 46,000 \times g) as described (8). Vector concentrations were analyzed by using an immunocapture p24-gag ELISA (Alliance, DuPont/NEN) (8).

Nucleic Acid and Protein Analysis. For RNA analysis, 20 μ g of total RNA were electrophoresed on 1% agarose gel/2.2 M formaldehyde, transferred onto nylon membranes (Hybond-N, Amersham Pharmacia), and probed according to standard methods

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; siGFP, cassette expressing siRNA specific for GFP; siHp53, cassette expressing siRNA specific for p53.

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with ^{32}P -labeled cDNA against GFP, actin, and human p53. For protein analysis, total protein was extracted with 50 mM Tris/150 mM NaCl/0.1% Triton X-100/0.1 mM DTT plus proteinase inhibitors. Protein (10–50 μg) was separated by 10% SDS/PAGE and immunoblotted according to standard methods with rabbit polyclonal antibodies against GFP (Abcam, Cambridge, U.K.) or β -actin (Sigma). Fluorescence-activated cell-sorter analysis was carried out as described (11).

PCR Detection. Viral and siRNA integration were detected by PCR analysis. Fifty to 100 ng of DNA were used in a 25- μl reaction. Primers spanning the H1-siGFP cassette were U3 forward (5'-CAAGGCAGCTGTAGATCTTAGCC-3') and U3 reverse (5'-GATCTTGTCTTCGTTGGGAGTG-3'). U3-H1 primers, which amplify the H1 portion of the H1-siRNA cassette, were U3 forward in combination with H1 promoter internal primer H1 reverse 5'-CGTACGGGCCCCGTGGTCTCATACGAAGTT-3'. The PCR conditions were 94°C denaturation for 3 min followed by 40 cycles of 94°C for 30 sec, 55°C for 40 sec, and 72°C for 50 sec. The GFP primers were GFP forward (5'-AAGTTCATCTGCACCAACCG-3') and GFP reverse (5'-TCCTTGAAGAAGATGGTGCG-3'). The conditions for PCR were as described above except PCR was carried out for 30 cycles.

Generation of Transgenic Mice. The basic methodology has been described by our laboratory (12). Briefly, 6-week-old B6D2 F₁ females were superovulated by injection of 5 units of pregnant mare serum gonadotropin (Sigma), followed 48 h later by injection of 5 units of human chorionic gonadotropin (Sigma) and mated with GFP-expressing transgenic males. Flushing the oviduct with FHM medium (Specialty Media, Lavellette, NJ) isolated two cell stage-fertilized eggs. Removal of the zona pellucida was achieved by acidic tyrode (Sigma) treatment. Transduction with lentiviruses was performed using 2,500 ng of p24 per ml in a volume of 5 μl of KSOM medium (Specialty Media) covered with light mineral oil. Forty-eight hours after transduction, blastocysts were transferred into the uteri of pseudopregnant CB6D2 F₁ females.

Results

Several groups have described the use of small nuclear RNA promoters (H1 and U6) for expressing siRNAs in mammalian cells (4, 13–15). We have used the H1 promoter (4) to drive expression of siRNA targeting GFP and human p53 (ref. 4; Fig. 1*a*). To verify that silencing is induced by the H1-siRNA constructs, we cotransfected siRNA-expressing plasmids together with a GFP-expressing plasmid into 293T cells. Silencing of GFP was detected starting 24 h posttransfection in cells transfected with the H1-siGFP plasmid but not with the H1siHp53 plasmid (Fig. 1*b*). We confirmed further that silencing of GFP was due to strong reduction in GFP mRNA levels (Fig. 1*c*) as compared with cells that were transfected with the irrelevant H1-siHp53 plasmid. Expression of human p53 was reduced in cells transfected with H1-siHp53 plasmid but not in cells transfected with H1-siGFP (Fig. 1*c*), indicating high target specificity.

We next designed lentiviral vectors expressing siRNA cassette (Fig. 2*a*) to test the hypothesis that siRNAs can be expressed efficiently from RNA viruses and induce specific silencing of target genes in transduced cells. Toward that end, we generated a lentivirus vector expressing siRNA directed against GFP (LV-siGFP, Fig. 2*a*). We inserted the H1-siRNA cassette in a unique restriction site at the U3 region of the LTR. The resulting provirus will acquire two copies of H1-siRNA cassette due to duplication of the LTR during reverse transcription. Thus, even in the case of a single integration event, target cells will be comprised of two transcription units of the siRNA cassette.

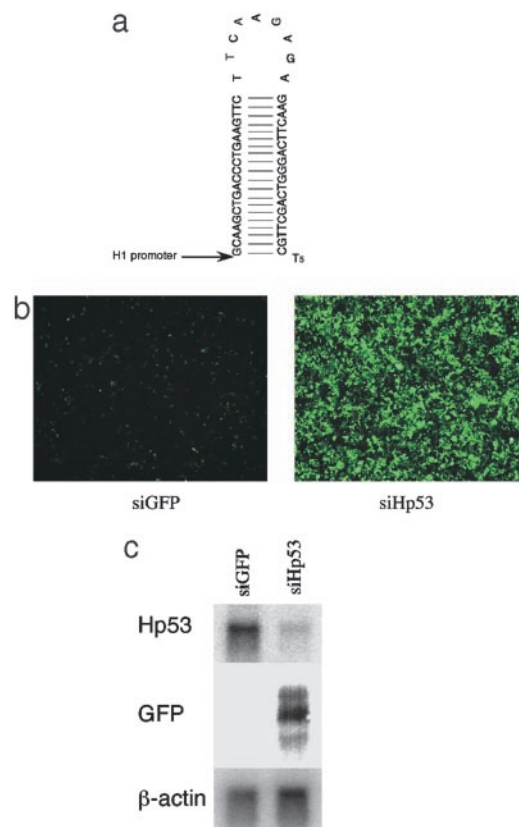


Fig. 1. Transfection by the siGFP plasmid. (*a*) Sequence and putative folding of siGFP oligonucleotide used to target the GFP gene. The 20-nt sequence of the target transcript separated by a short spacer (shown as a loop) from reverse complement of the same sequence is shown. The termination sequence consists of five thymidines (T₅). (*b*) Transfection of 293T cells with 0.2 μg of GFP-expressing plasmid together with 1 μg of a plasmid expressing siGFP or siHp53. A lack of GFP expression only in the presence of siGFP-expressing plasmid can be seen. Photographs were taken with a Zeiss microscope ($\times 5$). (*c*) Twenty micrograms of RNA prepared from transfected cells were electrophoresed on a denaturing gel and hybridized to radiolabeled GFP, human p53, and β -actin probes. Specific silencing of GFP and human p53 RNA was noticed only when the relevant specific siRNA was used.

We further asked whether an siRNA-expressing lentivirus could suppress expression of target genes in transduced cells. A stable GFP-expressing cell line (293T-GFP) was transduced with LV-siGFP virus at different multiplicities of infection. GFP fluorescence decreased proportionately with increasing multiplicities of infection (Fig. 2*b*). At a dose of 80 ng of p24 virus per 5×10^4 cells, GFP expression was barely detectable by fluorescence microscopy (Fig. 2*c*). Fig. 2*d* shows a concomitant reduction of GFP protein when probed with GFP antibodies. The effect on GFP was specific to LV-siGFP, because no reduction in GFP expression was observed when 293T-GFP cells were transduced with irrelevant LV-siHp53 virus. We thus conclude that lentivirus vector-generated siRNA can reduce the expression of the target gene successfully.

A powerful application of LV-siRNA viruses would be the ability to generate transgenic animals carrying siRNA cassette to induce an endogenous gene silencing. Our lab and others have shown previously that lentivirus vectors have the unique ability to generate transgenic rodents by *in vitro* transduction of fertilized eggs at different preimplantation stages (12, 16). We therefore were interested to test whether the LV-siGFP virus is able to silence GFP expression in GFP transgenic mice (TgGFP). We reasoned that by generating a TgGFP mouse that is also

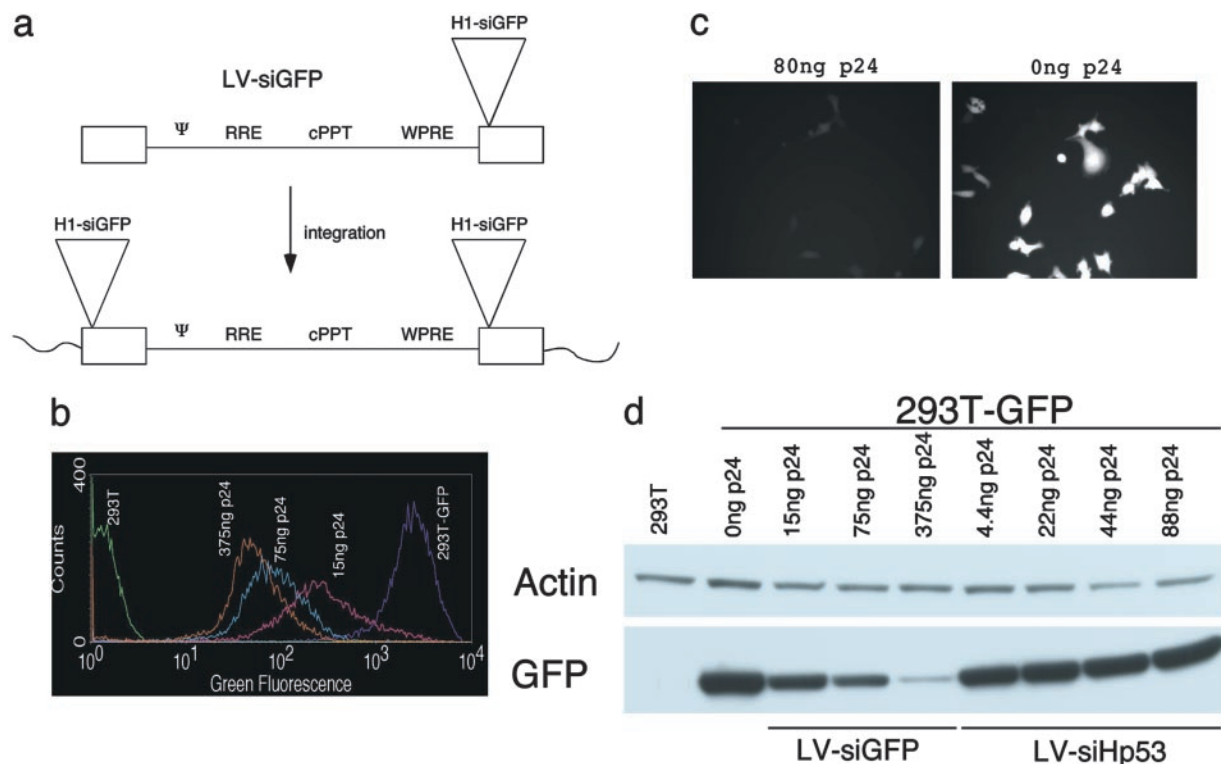


Fig. 2. LV-siGFP virus transduction. (a) Schematic drawing of lentiviral vector containing an siGFP cassette capable of generating siGFP-RNA. Shown also is the construct capable of generating two copies of siRNA after reverse transcription and integration. (b) Fifty thousand 293T cells were transduced with increasing amounts of LV-siGFP (15–375 ng of p24 per well). Fluorescence-activated cell-sorter analysis was performed to detect fluorescence. (c) Complete extinction of GFP expression by transduction of 80 ng (p24) of LV-siGFP virus in 5×10^4 293T-GFP-positive cells ($\times 30$). (d) 293T-GFP cells were transduced with increasing amounts of LV-siGFP (15–375 ng of p24 per well). Seventy-two hours later, whole-cell extracts were prepared, separated on 10% SDS/PAGE, and immunoblotted with polyclonal antibodies to GFP. As a control, 293T-GFP cells were also transduced with increasing amounts of LV-siHp53 and probed with GFP antibodies. β -Actin was used as a loading control.

transgenic for the H1-siGFP cassette, we should be able to show whole-body knockdown of GFP expression. Fertilized eggs were collected from females that were mated with TgGFP males that contained multiple copies of GFP on both alleles and transduced with LV-siGFP virus (ref. 17; Fig. 3a). Resulting progeny are hemizygous for the array of multiple copies of GFP. Reduction in GFP fluorescence in virus-treated blastocysts was noticed 48 h after transduction (Fig. 3a). The transduced blastocysts were transplanted into pseudopregnant females. Live pups were analyzed by fluorescence, and the presence of integrated lentivirus was confirmed by PCR with primers specific for the siGFP cassette (Fig. 3b). Two F₀ females (F₀-2 and F₀-4, Fig. 3b) were mated with wild-type males to obtain F₁ progeny. One pup (F₁-36) from female F₀-2 was found to be positive for the siGFP cassette (Fig. 3c). Interestingly, F₁-36 also showed an overall lower GFP fluorescence (Fig. 3d). Using TaqMan PCR with primers specific for lentivirus integration (18), we found that the average copy numbers of LV-siGFP for F₀-2 and F₀-4 were 13 and 21, respectively. The positive pup (F₁-36) had two copies of integrated lentivirus per cell. Considering that parental mice (F₀-2) had multiple copies of GFP, it is interesting to note that an average of two copies of siGFP per genome showed marked overall reduction of GFP in this mouse.

Embryos taken from pregnant female F₀-4 were examined for GFP expression, and one was found to have lower GFP expression. Of nine F₁ embryos analyzed, six were genotyped to be positive for the GFP array (Fig. 3e). Of these embryos number 6 and 8 were positive for integrated siGFP lentivirus (Fig. 3e), but embryo 8 was negative for GFP. Therefore, a silencing effect could be observed only in the F₁-6 embryo. Reduction of GFP

protein was observed in the F₁-6 embryo when probed with GFP antibodies (Fig. 3f). Using TaqMan PCR, we found that the copy number of lentivirus integration in the F₁-6 embryo was 10.

Discussion

We have described a methodology that can be used successfully to generate a large number of transgenic mice in which expression of specific genes can be down-regulated substantially. The technology builds on the use of lentiviral vectors for transgenesis combined with the use of RNAi to silence gene expression. Additionally, the ability of lentiviral vectors to transduce nondividing cells allows direct injection into a tissue or organ to knockdown the expression of specific genes (O.S., G.T., and I.M.V., unpublished data). Because lentiviral vectors integrate in the chromosome, the succeeding progeny is likely to inherit the provirus and express the appropriate genes. The present methodology allows us to generate (i) cell lines in which expression of specific gene(s) can be reduced extensively (Fig. 1b), (ii) “knocked-down” or hypomorphic mice with decreased expression of targeted gene product (Fig. 3d), and (iii) diminished expression of genes in brain and hematopoietic cells (data not shown). Although the work reported here deals with mice, lentiviral vectors have been shown previously to generate transgenic rats (16), and we predict that they can be used to generate even transgenic nonhuman primate animals to develop disease models.

Although the combination of lentiviral vectors with siRNA gene-silencing approaches shows great promise, a number of aspects of the technology require further development. At present there is no well defined set of rules for designing siRNA oligonucleotides. Because some candidate siRNAs work well and others do not, a panel of five to seven siRNAs must be tested empirically.

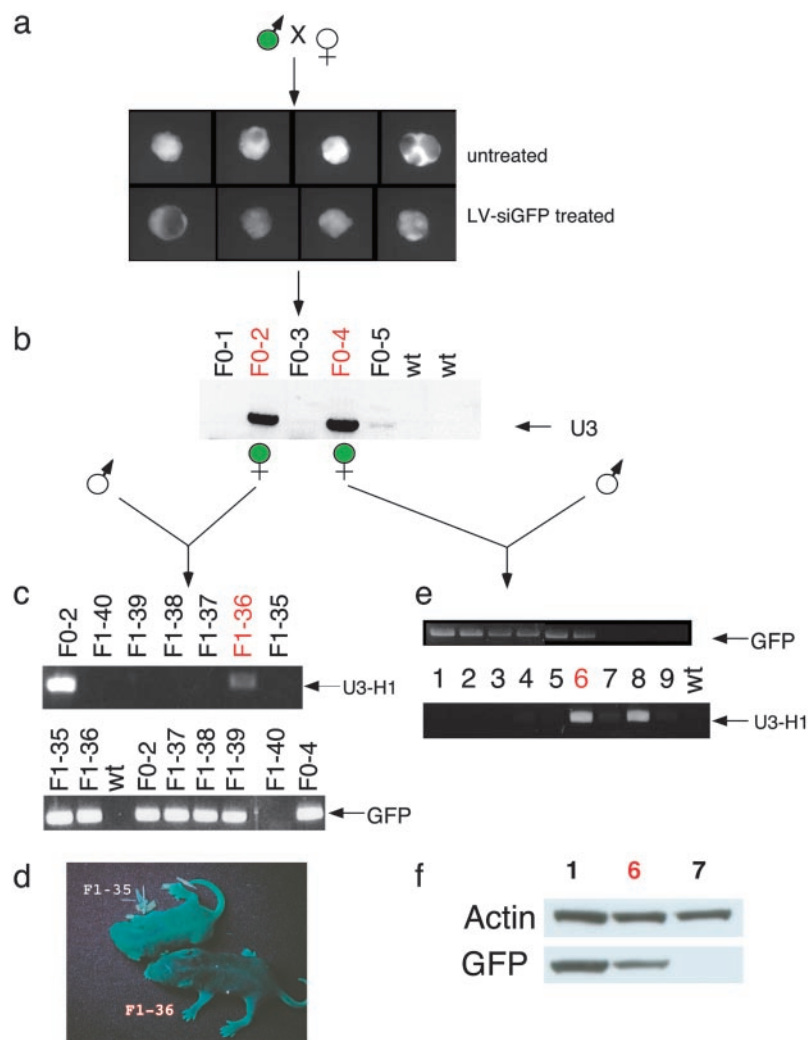


Fig. 3. Generation of GFP knockdown mice. (a) Decreased fluorescence of GFP in blastocysts transduced with LV-siGFP (♂, filled green depicts GFP transgenic male mouse; ♀, wild-type female). (b) F₀ animals were analyzed by PCR of genomic DNA using U3 primers. The U3 primers amplified the H1-siGFP cassette cloned in the U3 region in the LTR. The siGFP-positive F₀ mice (F₀-2 + F₀-4) are indicated in red. (c) PCR of genomic DNA from F₁ pups using U3-H1 primers. The combination of U3 forward and H1 reverse amplifies the H1 promoter portion of the H1-siGFP cassette. The positive F₁-36 pup is shown in red. Persistence of the GFP allele was confirmed by PCR using GFP primers. (d) Shining with fluorescence lamp of F₁ littermate pups at day 3 of age (pictures were taken with a digital camera). (e) Embryonic day-13 embryos were harvested from pregnant female F₀-4, and GFP sequences were detected using GFP-specific PCR primers. F₁ embryos positive for integrated copies of the siGFP cassette using U3-H1 primers are shown. The F₁-6 embryo positive for both GFP and siGFP is shown in red. (f) Protein extracts from embryonic day-13 embryos were analyzed by probing with a GFP antibody. Shown are embryos 1, 6, and 7. Reprobing with β -actin antibody was used as a loading control.

Furthermore, it is not clear what molar ratio of siRNA molecules to target gene mRNA is required for effective knockdown or whether this parameter will be targets-specific. It will be useful to know how much siRNA is required to knockdown or knockout expression from a one-copy gene, which most likely will be the case for most functional genomics studies. Lentiviral vectors can be modified where multiple siRNAs are expressed from a single provirus. It is also not clear whether expression from integrated lentiviral vectors will be silenced in certain progeny or tissues. Transcriptional shut-off from integrated retroviral DNA has been observed (19), whereas the problem seems to be less acute with lentiviral proviruses. Despite these caveats and limitations, we believe the use of lentiviral vectors capable of expressing siRNA will prove to be a very useful tool to study gene function. Furthermore, if the expression of siRNA in lentiviral vectors can be regulated, it can be extremely useful to study development and differentiation. Finally, for gene-therapy experiments, it will be very useful to have animal model systems. The ease and convenience of deleting gene

function combined with the ability to make transgenic animals with the appropriate mutant gene will be very beneficial for proof of concept. Furthermore, the transgenic mice containing the actual mutant gene will also be very useful to study immunological consequences of the transgene introduced by gene-delivery vectors. The technology described here will also be very helpful for gene therapy of genetic diseases due to gain of function such as Huntington's disease. In summary, we describe the development of a methodology to generate a large number of cell lines, tissues, and whole animals where the expression of targeted genes can be reduced substantially to influence biological function.

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